Current Microbiology Vol. 52 (2006), pp. 305–309
DOI: 10.1007/s00284-005-0303-8

Current Microbiology
An International Journal
© Springer Science+Business Media, Inc. 2006

Identification and Characterization of an *Aeromonas salmonicida* (syn *Haemophilus piscium*) Strain That Reduces Selenite to Elemental Red Selenium

William J. Hunter,[1] L. David Kuykendall[2]

[1]USDA–ARS, 2150-D Centre Avenue, Fort Collins, CO 80526-8119, USA
[2]USDA–ARS, BARC-W, Building 004, 10300 Baltimore Avenue, Beltsville, MD 20705, USA

Received: 15 September 2005 / Accepted: 12 October 2005

Abstract. A bacterium that reduces toxic and mobile selenite to insoluble elemental selenium (Se^0) was isolated from a laboratory scale permeable reactive biobarrier. Biochemical tests and 16S rRNA gene sequence alignment identified the isolate as *Aeromonas salmonicida.* Two colony types were isolated, one more resistant to selenite than the other. Both grew on agar plates containing 16 mM selenite, although the colony diameter was reduced to 8% of controls with the small colony type and to 18% with the large colony type. Further study was done with the large colony type. In anaerobic culture, this bacterium was able to use nitrate as a term electron acceptor but not selenate or selenite. In aerobic culture, when no nitrate was present, early log phase cells removed selenite at a rate of 2.6 ± 0.42 μmol SeO_3^{-2}/mg protein/day. Reduction was retarded by 25 mM nitrate. Mutants with a diminished ability to reduce selenite to Se^0 also had a reduced ability to reduce nitrate to nitrous oxide. This bacterium, or perhaps its enzymes or DNA, might be used to remove selenite from contaminated groundwaters.

Selenite is highly soluble and mobile in groundwater and is more toxic than other forms of selenium [4]. Permeable reactive biobarriers (PRB) inoculated with bacteria that reduce selenite to elemental selenium (Se^0) may provide a means of removing selenite from water. Se^0 is insoluble; therefore, its bioavailability is greatly reduced relative to that of selenite. In most groundwaters, microbial activity is limited by the amount of substrate or electron donor available. PRBs function by stimulating microbial growth and activity by providing an electron donor [6, 16]. Within PRBs there are several processes that can detoxify selenite [1]. Microorganisms may convert it into Se^0 or methylate it. Also, biologically reduced compounds in the PRB may cause the abiotic reduction of selenite. Only a few bacteria have been characterized that are capable of reducing soluble selenium oxyanions to Se^0. This article describes the characterization and presumptive identification of a bacterial isolate that reduces selenite to elemental red selenium.

Correspondence to: W.J. Hunter; *email:* william.hunter@ars.usda.gov

Materials and Methods

Media. Media used were nutrient broth and agar obtained from Difco/Becton Dickinson (Sparks, MD, USA), NWRI media [10] and an HM-salts media (pH 6.7) that contained 30 mM glycerol, 1.3 g *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid, 1.1 g 2-(*N*-morpholino) ethanesulfonic acid, 27 mg iron-ethylenediaminetetraacetic acid, 88 mg $MgSO_4$, 13 mg $CaCl_2$, 250 mg Na_2SO_4, 320 mg NH_4Cl, 125 mg Na_2HPO_4, 0.5 mg H_3BO_3, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.01 mg $CuCl_2 \cdot H_2O$, 0.01 mg MoO_3, and 0.1 mg $ZnCl_2$ L^{-1}. Agar, 15 g L^{-1} was added to make solid media, and media were supplemented with Na_2SeO_4 or Na_2SeO_3 when indicated.

Isolation. The bacterium was isolated from a laboratory PRB that contained soybean oil as an electron donor. The PRB, inoculated with a soil extract and supplied with water containing 10 ppm selenite-Se, was effective at removing selenite from water via reduction to Se^0 [8]. After 11 weeks use, the PRB was disassembled and culturable microorganisms that were dominant in the PRB were isolated by suspending a 1-g sample of the barrier in 9 mL of NWRI media. Liquid from this suspension was used to inoculate a 10^{-2} to 10^{-6} tube dilution series using NWRI media as the diluent. Tubes were incubated under

helium for 1 week at 22°C. Next, 100 μL from each tube was spread onto NWRI agar containing 0.5 mM sodium selenate and incubated under helium until colonies developed. Strain C278 was isolated from the 10^{-5} dilution tube.

Identification and characterization of the isolate. Cells, incubated on NWRI plates for ~7 days at 22°C, were examined for cell and colony morphology. Reactions on the API-20E® test strips (bioMérieux Inc., Hazelwood, MO, USA), incubated per the manufacture's directions, was entered into the API database for an initial identification. Cells were also sent to MIDI Labs (Newark, DE, USA) for 16S rRNA (1540 bp) gene sequence determination. The sequence was analyzed on the Applied Biosystem's MicroSeq™ database and on the BLAST blastn database at the National Center for Biotechnology Information.

Effect of selenite and selenate on growth. Inhibition studies were conducted using NWRI agar supplemented with sodium selenate or selenite as indicated. Log phase cells from NWRI broth were streaked onto the plates and the plates incubated for 5 days at 28°C. Isolated colonies in each group were measured with a micrometer-equipped dissecting scope.

Selenate and selenite assay. Selenate samples, 400 μL, were mixed with 400 μL of concentrated HCl and heated at 95°C for 30 min. Samples were cooled to 4°C and 200 μL of 1 M glycine, 1.00 mL of a 30% NH_4OH solution (28–30%, 26° Baumé), 400 μL of hydroxylamine-EDTA solution (25 g L^{-1} hydroxylamine hydrochloride and 9 g L^{-1} disodium ethylenediaminetetraacetic acid), and 1.00 mL of 2,3-diaminonaphthalene solution (1 mg mL^{-1} in 0.1 M HCl) added. The mixture was shaken in a 50°C water bath for 30 min, cooled, and absorbency read at 378 nm. The assay was modified from Greenberg et al. [10]. For selenite, 200 μL of sample was mixed with 1.8 mL of water, 400 μL of hydroxylamine-EDTA solution, and 1.00 mL of 2,3-diaminonaphthalene solution. This mixture was shaken, incubated, and its absorbency determined as described for selenate.

Dimethylselenide and dimethyldiselenide production. Culture bottles were 155-mL bottles containing 50 mL of nutrient broth supplemented with 4 mM Na_2SeO_4 or Na_2SeO_3. Bottles, stoppers, and base media were autoclaved and Na_2SeO_4 and Na_2SeO_3 filter sterilized. Bottles were inoculated with 1 mL of inoculum solution prepared by suspending a loop full of cells in 5 mL of a 0.85% NaCl (saline) solution from 4-day-old nutrient agar plate cultures. Inoculated bottles were sealed with Teflon® lined stoppers and incubated under air at 22°C and 100 rpm. On day 4, atmospheric samples were analyzed as described in Hunter and Kuykendall [7].

Selenite and selenate as electron acceptors. Media was HM-salts media, 10 mL in Hungate tubes, supplemented with 4 mM sodium selenite, 10 mM sodium selenate, or 10 mM sodium nitrate. Cultures were initiated by adding ~100 μL of log phase cells and incubating cell at 28°C under nitrogen for 2 weeks. Growth was estimated by turbidity (A_{660}) and protein measurements. For protein, cells were digested [12] and protein estimated by the Lowry [13] or by the biuret procedure [5] using bovine serum albumin as a standard.

Denitrification assay. Serum bottles, 155 mL, containing 25 mL of HM-salts media with 50 mM glycerol and 10 mM nitrate, were inoculated and incubated at 28°C under He supplemented with ~2% acetylene. Samples of the atmosphere were collected at intervals and N_2O estimated as described previously [6].

Selection of "orange mutants". Cells resistant to rifampicin were obtained by streaking cells onto NWRI agar supplemented with 150 mg mL^{-1} rifampicin and incubating at 22°C until colonies formed. Clones were isolated and used for the next selection where 2×10^8 CFU of early log-phase cells were exposed to 30 W of 254 nm light at a distance of 60 cm. Exposure times were adjusted to yield a 1% survivor rate. Survivors were plated onto NWRI media containing 4 mM selenite and colonies that produced orange rather than dark red colonies isolated as "orange mutants." Cells were plated onto NWRI media containing rifampicin to confirm that the cells were not contaminates.

Statistical comparisons. Statistical comparisons are standard error of the mean computations, made using the Instat® computer program (GraphPad Software Inc.).

Results and Discussion

Characterization and identification. On nutrient agar colonies of strain C278 were circular, entire, raised, glistening, cream-colored, and odor producing. Gram reaction by KOH was Gram negative. No pigment was produced at either 22°C or 37°C after growth for 1 week on NWRI or on nutrient agar. Growth on nutrient agar at 37°C was about the same as growth at 22°C yielding colonies that were ~1 mm in diameter after 24 h. Unshaken broth tube cultures produced a uniform turbidity, and a motility test was positive. Microscopic examination showed that cells of strain C278 was rod shaped and motile. Biochemical tests show that C278 was positive for arginine dehydrolase, cytochrome oxidase, gelatinase, nitrate reductase, glucose and mannitol fermentation/oxidation, and was negative for ß-galactosidase, H_2S production, indole production, lysine decarboxylase, nitrogen production, ornithine decarboxylase, tryptophan deaminase, urease, and Voges Proskauer. Cells grew on citrate, rhamnose, and saccharose but not on arabinose, amygdalin, melibiose, inositol, sorbitol, or 6% NaCl. Using these characteristics and the API database, it is likely that C278 is an *Aeromonas* species. For a more rigorous identification, 1540 base pair 16S rRNA gene sequence alignment was employed. The sequence (GenBank Accession #AY910844) suggested a relationship to *Haemophilus piscium, A. bestiarum*, and *A. salmonicida* (Fig. 1), with the closest 16S rRNA match occurring between strain C278 and *H. piscium*. However, *H. piscium* is generally considered to be an atypical achromogenic *A. salmonicida* [15 and others]. Generally, bacteria of the same species will show a percent genetic difference of 0.5% to 3.0% [14]. Strain C278 showed a 0.03% 16S rRNA genetic difference from *A. salmonicida.* We consider C278 to be an atypical *A. salmonicida*. The strain was atypical in that it was able to grow at 37°C, was motile, and produced no pigment.

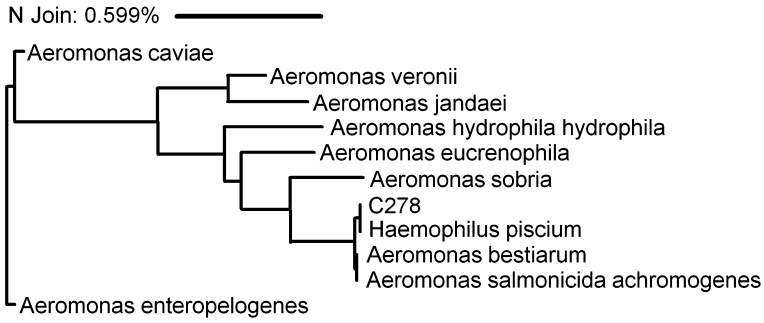

Fig. 1. Neighbor-joining tree displaying the interspecies relationship between strain C278 and other related strains. Neighbor-joining tree was developed by MIDI Labs (Newark, DE, USA). Relationships are based on the MicroSeq database. Scale shows percentage divergence.

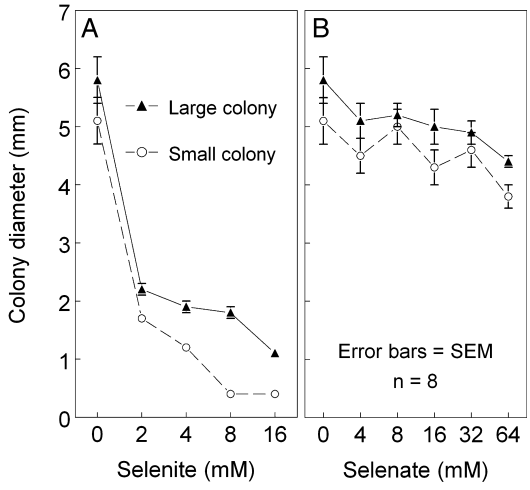

Fig. 2. Effect of selenite (A) and selenate (B) on the colony size of *A. salmonicida* strain C278.

When supplemented with 4 mM selenite C278 yielded colonies with deep red centers, indicating that selenite was being reduced to elemental red selenium, Se^0 [1]. However, there was no evidence for the reduction of selenite to dimethylselenide (DMS) or dimethyldiselenide (DMDS). When strain C278 was incubated in sealed bottles, neither DMS nor DMDS was detected. The presence of 16 mM selenite in NWRI agar reduced but did not prevent growth. Two colony types formed on agar plates that contained selenite. About 95% of the colonies were "small colonies" (C278s) and about 5% were "large colonies" (C278L). When C278s was streaked onto agar containing 4 mM selenite, both large and small colony types developed, but when C278L cells were streaked only large colonies formed. Selenite sensitivity affected colony size. C278s was more sensitive to selenite than was C278L (Fig. 2A). Selenate inhibited growth far less than did selenite (Figure 2B).

Under anaerobic conditions, the growth of strain C278L was stimulated by 10 mM nitrate, and cultures of C278L produced nitrous oxide when incubated under a helium/acetylene atmosphere showing that it can use nitrate for respiration. However, neither 10 mM selenate nor 4 mM selenite could serve as a respiratory electron acceptor (Table 1). When both nitrate and selenite were present a reddish color developed, indicating the reduction of selenite to Se^0. In broth culture under aerobic conditions, the addition of 10 mM selenate to the growth media had no significant influence on the growth of C278L, whereas the addition of 4 mM selenite to the broth media reduced growth. When 2.5 mM selenite was incorporated into the media, heavy growth was observed, a deep red color developed, and the amount of soluble selenite present declined at a rate of about 0.28 mM day^{-1} (Table 2). The specific activity of the removal was highest on day 1 at 2.6 ± 0.4 μmol SeO_3^{-2} mg $protein^{-1}$ day^{-1}. High levels of nitrate interfered; when 25 mM nitrate was present in the media measurable amounts of selenite were not removed.

Table 1. Effect of nitrate, selenate and selenite on the anaerobic growth of strain C278L

Treatment	Turbidity (mA_{660})	Protein (μg ml^{-1})
None	18.2 ± 8.7	70 ± 5
10 mM NO_3^-	38.4 ± 4.1	110 ± 8
10 mM SeO_4^{-2}	12.6 ± 0.5	44 ± 1
4 mM SeO_3^{-2}	18.5 ± 6.2	60 ± 2

Bacteria can reduce selenite to Se^0 by several mechanisms. In *Thauera selenatis* selenite is reduced to Se^0 by a periplasmic nitrite reductase [3]. In contrast, an *Escherichia coli* mutant that possessed a defective nitrite reductase was able to accumulate Se^0, suggesting that a different system was involved [2]. Studies with mutants of C278L suggest a linkage between selenite reduction and denitrification like that of *T. selenatis*. We exposed C278L to UV irradiation and isolated mutants with a weakened ability to reduce selenite to Se^0. This reduced capability was evidenced by the inability of these colonies to accumulate enough Se^0 to form the dark red colonies characteristic of the wild-type parent. Instead, these mutants accumulated only enough Se^0 to form

Table 2. Growth and selenite removal from aerobic cultures of C278L cultured in shaken flasks containing HM salts media supplemented with 0.1% yeast extract

Incubation time (Day)	SeO_3^{-2} removed (mM)	NO_3^- present (mM)	Biuret Protein (mg/L)	Removal rate (μMole SeO_3^{-2} mg protein^{-1} day^{-1})
Without nitrate				
0	0 ± 0.1	0.2 ± 0.0	−20 ± 12.5	N/A
1	0.4 ± 0.0	0.8 ± 0.1	148 ± 25	2.6 ± 0.42
2	0.6 ± 0.0	0.8 ± 0.1	212 ± 27	1.35 ± 0.23
3	0.9 ± 0.0	0.4 ± 0.0	468 ± 53	0.65 ± 0.11
4	1.1 ± 0.1	0.3 ± 0.0	660 ± 66	0.33 ± 0.07
With 25 mM nitrate				
0	0 ± 0.2	19.9 ± 0.9	−76 ± 25	N/A
1	−0.1 ± 0.1	23.8 ± 0.3	58 ± 12	0 ± 0
2	0.0 ± 0.0	23.9 ± 0.3	134 ± 20	0 ± 0
3	0.0 ± 0.1	23.6 ± 0.2	175 ± 12	0 ± 0
4	0.0 ± 0.1	23.8 ± 0.2	208 ± 15	0 ± 0

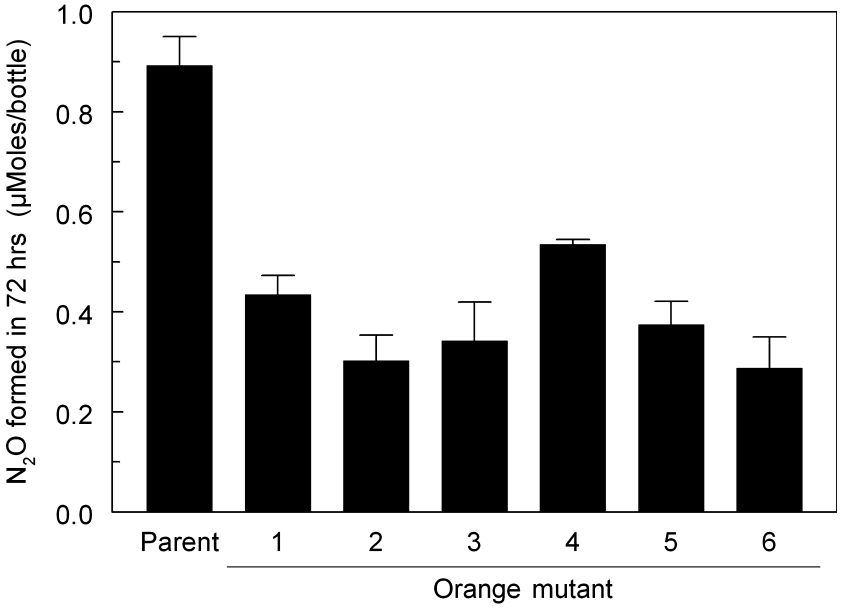

Fig. 3. Denitrification, measured by the accumulation of N_2O, by the wild-type parent and by mutant strains of C278 that form orange-colored colonies on agar supplemented with selenite.

orange-colored colonies ''orange mutants'' when grown on media supplemented with selenite. Anaerobic incubations of these orange mutants, with nitrate as term electron acceptor, showed that they also had a reduced ability to reduce nitrate to nitrous oxide (Fig. 3). The concurrent lessening of both abilities suggests that some element of the same pathway is associated with the reduction of nitrate to nitrous oxide and selenite to Se^0, the most likely candidate being nitrite reductase.

The reduction of metals and metalloids by microorganisms has broad biological and environmental importance. The identification of bacteria capable of reducing selenate and/or selenite to Se^0 can provide useful information that may help in the detoxification or removal of this sometimes environmental toxin from contaminated groundwaters and industrial effluents. At present, not much is known about the impact aeromonads may have in the reduction of environmental metals and metalloids. These bacteria, or perhaps their enzymes or DNA, may prove to be useful tools for the remediation of contaminated groundwaters. Additionally, we need to know what bacterial populations may increase when biobarriers are used to remediate soluble selenium compounds and the impact that these bacteria may have on groundwater or drinking water quality. *Aeromonas* species are widely disseminated in soil and in aqueous environments and are frequently present in drinking water supplies [9]. It may not be desirable to increase the

population of *Aeromonas* sp. in waters that are used for human or animal drinking supplies because of the link between some *Aeromonas* species and animal, and possibly human, disease [11].

ACKNOWLEDGMENTS

The authors are thankful to Robin Montenieri, Tammy Stockett, and Joe Kelly for providing expert technical assistance. Manufacturer and product brand names are given for the reader's convenience and do not reflect endorsement by the U.S. government. This article was the work of US government employees engaged in their official duties and is exempt from copyright.

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